Anticancer activity of *Tabernaemontana catharinensis* extract obtained by supercritical fluid extraction

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RESUMO: **Atividade anticancerígena de extrato de *Tabernaemontana catharinensis* obtido por extração supercrítica.** No presente trabalho a atividade anticancer do extrato bruto de *T. catharinensis* foi avaliada. O extrato bruto foi obtido utilizando CO_2 supercrítico na pressão de 300 bar e temperatura de 55 ºC; etanol foi usado como co-solvente (4.6% massa). A atividade anticancer foi testada contra sete linhagens de tumores humanas: pulmão, mama, mama expressando o fenótipo resistente a múltiplas drogas, melanoma, cólon, próstata e rim. O extrato bruto foi fracionado e a fração alcaloídica foi analisada por cromatografia em camada delgada (CCD) e cromatografia a gás com detector por ionização em chama (CG-DIC). A CG-DIC da fração alcaloídica indicou a presença dos alcalóides indólicos voacangina e coronaridina. O extrato bruto de T.catharinensis apresentou atividade anticâncer a partir de 0.25 μg/mL com efeito citostático e citolítico, dependente da concentração e sem seletividade entre as linhagens celulares utilizadas.

Palavras-chave: *Tabernaemontana catharinensis*, alcalóides indólicos, extração supercrítica, atividade anticancer

ABSTRACT: In the present work the anticancer activity of crude supercritical fluid extract from *T. catharinensis* was evaluated. The crude extract was obtained using supercritical CO_2 at pressure of 300 bar and temperature of 55 ºC; ethanol was used as cosolvent (4.6% mass). The anticancer activity was tested against seven human cancer cell lines: lung, breast, breast expressing the multidrug resistance phenotype, melanoma, colon, prostate, and kidney. The crude extract was fractionated and the alkaloidal fraction was analyzed by thin-layer chromatography (TLC), and gas-chromatography/flame ionization detector (GC-FID). The GC-FID of the alkaloidal fraction indicated the presence of the indole alkaloids voacangine and coronaridine. The crude extract of *T.catharinensis* has anticancer activity that begins at 0.25 μg/mL and exhibits cytostatic and cytolytic effects concentration dependent and not selective for the cell lines used.

Key words: *Tabernaemontana catharinensis*, indole alkaloids, supercritical fluid extraction, anticancer activity

INTRODUCTION

The discovery of new drugs starting from tropical plants has stimulated the research in native plants in the last years. Extracts and many isolated compounds from these plants are used for elaboration of new drugs. For instance, the alkaloids vinblastine and vincristine extracted from *Catharanthus roseus* have been widely used in the treatment of various human cancers such as in the treatment of acute leukemia, and many other lymphomas (Singh et al, 2001; Chiang et al, 2004; Plasschaert et al, 2004). *Tabernaemontana catharinensis* (syn. *Peschiera catharinensis* A.DC.) is a species belonging to the *Apocynaceae* family that is rich in indole alkaloids. According to Leeuwemberg (1994), this species has also been denoted as *T. affinis, T. australis, P. australis, T. hilariana, T. hybrida, T. acummiata, P. albidiflora,* and *T. salicifolia*. Pharmacological studies on the crude extracts from *T. catharinensis* have demonstrated its antimicrobial, anti-inflammatory, antitumor, and analgesic activities (Pereira et al, 2003; Rates et al, 1993; Spitzer et al, 1995; Pereira, 1999). The effective actions of these extracts are associated with the presence of indole alkaloids. In the study developed by Rates et al (1995), the antitumoral activity of crude extracts of *T. catharinensis* seeds and leaves was demonstrated. The antitumoral activity was evaluated, *in vivo*, against Sarcoma 180 and Ehrlich's carcinoma; this activity was associated to the alkaloids coronaridine and olivacine. Recently, it was reported that the crude extract and its isolated indole alkaloids exhibited significant tripanocidal and antileishmanial activities (Pereira, 1999; Delorenzi et al, 2001). In these works, the extracts were obtained by Soxhlet procedure with one of the following solvents: chloroform, petroleum ether, or ethanol.

Due to the co-extraction of several substances the fractionation of extracts obtained by Soxhlet and any other conventional processes, such as, leaching, percolation, elution, and so on is a cumbersome process. In addition, a significant part of the active or target compounds is lost. The alternative process denoted as supercritical fluid extraction (SFE) is associated with the easy removal of the solvent from the extract. Other advantage of SFE is the selectivity of the supercritical solvent at a given condition of

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pressure and temperature for a specific class of chemicals such as terpenes, alkaloids, etc. The solvent power of the supercritical solvent is directly related to its density. In the supercritical phase, small changes in pressure or temperature can produce large variation in the solvent density, thus, influencing its solvation power. In general, a large increase in solvent density decreases its selectivity. Since, carbon dioxide is non-toxic and has low critical temperature (31.4 °C) and intermediate critical pressure (78.3 bar), it is the standard solvent for applications related to obtaining natural extracts. Unfortunately, carbon dioxide has a limited solvation power, so it is virtually unable to solubilize the indole alkaloids present in *T. catharinensis*. This behavior can be modified by the addition of a cosolvent, such as ethanol, isopropyl alcohol, water, etc.

Pereira et al (2003) obtained extracts from *T. catharinensis* using supercritical CO_2 + ethanol, and fractionated the crude extract (CE) obtaining three fractions: the alkaloidal fraction (AF), the hexanic fraction (HE), and the aqueous fraction (AqE). The antioxidant and antimycobacterial activities of these fractions (AF, HE, and AqE) including the crude extract (CE) were evaluated. The antioxidant activity of the crude extracts ranged from 70% to 88%, while that of the alkaloidal fraction ranged from 72% to 88%. These results indicate that the crude SFE extracts of *T. catharinensis* can be used as antioxidant. On the other hand, the highest antimycobacterial activity against *M. tuberculosis* was detected in the alkaloidal fraction (MIC = 128 mg/mL). In contrast, the crude extracts had low antimycobacterial activity (MIC≥ 512 µg/mL). In another study using the alkaloidal fraction of SFE extracts from *T. catharinensis*, the leishmanicidal effect and cytotoxicity to macrophages were evaluated (Soares et al, 2003). The results showed that the alkaloidal fraction (10 and 100 µg/mL) inhibited 20-26 and 80-100%, respectively, of parasite growth. Beside that the AF was found to be non-toxic for macrophages.

These works suggest that the extracts and fractions obtained by SFE kept the biological activities of this specie. Therefore, the objective of this work was to evaluate the anticancer activity of *T. catharinensis* extract obtained by supercritical fluid extraction against seven human cancer cell lines. The major compounds of the fractionated extract were identified and quantified by CG-FID.

MATERIAL AND METHOD

Preparation and characterization of the extract

The extract was obtained using thin branches and leaves from *Tabernaemontana catharinensis* collected by FIOCRUZ (RJ, Brazil) from Guará at the municipality of Campinas (SP, Brazil). Voucher specimen is deposited in the Botanic Garden of Rio de Janeiro (RJ, Brazil), under Nº RB 352.541. The raw material was dried at ambient conditions under shadow and subsequently triturated. Afterwards, the *raw* material was transferred to LASEFI – DEA / FEA – UNICAMP, conditioned under vacuum in plastic bags, and stored in a domestic freezer (Metalfrio, double action, São Paulo, SP) at –10 °C. The size distribution of the particles was determined using a mechanical agitator (Abrosinox, model Granutest, Santo Amaro, SP) with the rheostat set at 10 during 10 minutes; sieves of meshes (Standard Tyler series) 24, 32 and 48 were used.

Supercritical Fluid Extraction Procedure.

The experimental run was conducted using a supercritical fluid extraction unit containing an extraction cell of approximately 221×10^{-6} m^3 (length of 37.5×10^{-2} m and inside diameter of 2.74×10^{-2} m) and maximum pressure of 400 bar described by Pasquel et al (2000). The extract was obtained using supercritical CO_2 + ethanol as cosolvent (4.6% mass). The data was taken at 300 bar and 55 °C, using the methodology described by Pereira et al (2004). The bed was formed inside the extraction cell with *T. catharinensis*. About $(72\pm1)\times10^{-3}$ kg of raw material were used. Extract samples were collected in 100×10^{-6} m^3 glass flasks every 60 min. and typical runs continued for 540 min. Afterwards, the CO_2 + ethanol flow stopped and the system depressurization began and took 45 minutes. The cosolvent was eliminated from each extract sample using a vacuum oven (Napco, model 5831 Winchester, USA), at ambient temperature (< 25°C) for 24 hours. The yield (%) was calculated as the ratio of the extract mass (without cosolvent) to the initial mass of dried *T. catharinensis*. The overall extraction curve (OEC) was obtained using the accumulated yield against the time of extraction.

Analysis of the SFE extract

The identification and quantification of the indole alkaloids required the following procedure: fractionation of the crude extract to obtain the indole alkaloidal fraction (Delorenzi, 1998) that was analyzed by thin layer chromatography followed by gas chromatography with flame ionization detector (Cardozo, 1997).

Fractionation of the SFE extract

The SFE extract (CE) was dissolved in HCl 5% (fumigating 37%, Merck, P.A.) and washed three times with hexane (Merck, P.A., lot K26803774934), to remove wax and lipidic compounds. The aqueous extract was alkalinized with NH_4OH (25%, Merck, P.A.) and washed three times with chloroform (Merck, P.A., lot K28335045). The organic fraction (AF alkaloidal fraction) was evaporated using a rotatory evaporator (Laborota, model 4001, Viertrieb, Germany), with vacuum control (Heidolph Instruments GMBH, model Rotavac control, Viertrieb, Germany), and thermostatic bath at 40°C of the thermostatic.

Thin-layer chromatography (TLC).

The organic fraction (AF) was analyzed by TLC using silica plates (60-PF254, Merck 20 × 20 cm, 0.25

mm of height, lot 940378601) developed with a mixture 90:10 of chloroform (Ecibra, Chromatographic grade, lot 90466l) and methanol (Merck, Chromatographic grade, lot K26224109909). The plates were revealed in Dragendorff, the specific reagent for visualization of the alkaloids.

Gas chromatography with flame ionization detector (GC-FID)

The alkaloidal fraction (AF) was analyzed in a Gas-chromatographer with Flame Ionization Detector (GC-FID, Shimadzu, model 17A, Kyoto, Japan), equipped with a capillary column of fused silica DB-5 (J&W Scientific; 30 m × 0.25 mm × 0.25 μm, Folsom, USA). The carrier gas was helium at 1.7 mL/min (99.99% purity, White Martins Gases Industriais). The injector and detector temperatures were 250 °C and 280 °C, respectively. The temperature programming was 100 °C (5 min), 100 – 280 °C, 10 °C/min; 280 °C (10 min). The sample split ratio was 1/30. Samples of 1μL of extract diluted in ethyl acetate (5×10^{-6} kg of extract diluted in 1×10^{-6} m^3 ethyl acetate, EM Science, lot 3903991, chromatographic grade) were injected. The identification and quantification of the substances were based on comparison of AF's chromatogram with i) literature (Delorenzi, 1998; Cardozo et al, 1997; Van Beek, 1984) and ii) standards: coronaridine (79.55%), voacangine (80.99%) and isovoacangine (100%).

Anticancer Activity

The anticancer activities were evaluated for two samples of the crude extract collected over the extraction time intervals of 60-120min and 540-585 min of SFE. The analysis was carried out according to the methodology developed by the National Cancer Institute (NCI-USA) in which seven human cancer cell lines are involved: UACC62 (melanoma), MCF7 (breast), NCI.460 (lung), OVCAR (ovary), PC03 (prostate), C786.0 (kidney), and NCIADR (breast expressing multidrug-resistance phenotype). These cells were cultured in 25 cm^3 flasks (Nunc Brand Products, Roskilde, Denmark) containing 5 mL of RPMI 1640 (Gibco BRL, Life Technologies, São Paulo, SP) with 5% fetal bovine serum (Gibco BRL, Life Technologies). Stock cultures were kept in liquid nitrogen and were unfrozen for use when the ones being tested reached the twentieth serial passage. The samples of *T. catharinensis* extract were tested at four concentrations (0.25, 2.5, 25, 250 μg/mL), and each concentration were studied in triplicate wells. Microtiter plates containing cells were pre incubated for 24 h at 37 °C in order to allow their stabilization prior to the addition (100 μL) of the test substance (crude extract). The plates were incubated with the test substance for 48 h at 37°C and 5% CO_2. The cells were fixed by means of protein precipitation with (50%) trichloroacetic acid (TCA) (Sigma Chemical Co.) at 4 °C (50 μL/well, final concentration 10%) for 1h. The supernatant was then discarded, and the plates were washed five times with tap water. The cells were stained for 30 min with 0.4% of the SRB (Sigma Chemical Co.) dissolved in 1% acetic acid (50 μL/well) (Sigma Chemical Co.) and subsequently washed four times with 1% acetic acid to remove unbound stain. The plates were air-dried, and bound protein stain was solubilized with 150 μL/well of 10mM Trizma buffer (Sigma Chemical Co.). The optical density was read on an automated spectrophotometer plate reader at 540 nm. The percentage of growth inhibition was related to different concentrations of the test substance in graphic modes (Skehan et al, 1990; Monks et al, 1991).

RESULT AND DISCUSSION

The overall extraction curve (OEC) is shown in Figure 1. The extraction yield increased up to 240 min. of extraction (R_{240} = 0.51%), remained approximately constant (R_{540} = 0.53%) up to the end of the process at 540 minutes. During the depressurization of the system, the yield increased to its final value (R_T = 0.70%). Other results and considerations about the effects of operating pressure and temperature on the yield and chemical composition of the SFE extract were discussed by Pereira et al (2004).

The alkaloidal fraction represented 44.3% of the crude extract. The GC-FID of the alkaloidal fraction (AF) indicated that the major compounds present in the SFE extract were the indole alkaloids voacangine and coronaridine (Figure 2). The quantification of these alkaloids was determined for each sample collected every 60 min. Table 1 shows the mass of crude extract, the percentages of alkaloidal fraction in the crude extract in the crude extract (AF/CE), and the content of voacangine in the alkaloidal fraction (V/AF) for the samples collected over the extraction time intervals of 60-120 and 540-585 min.

Although, not all the chemical constituents of the crude extract (CE) were identified, this extract contained alkaloids (Pereira et al, 2004), and large amount of alkaloids was obtained in the sample collected during 540 – 585 min of extraction (in the depressurization step). However, the content of voacangine in the AF obtained during the depressurization was smaller than that in the sample collected over the extraction time interval of 60 – 120 min. These results indicate that besides voacangine and coronaridine other alkaloids were extracted but not identified by CG-FID. Pereira et al (2004) identified by IR, ^1H NMR and ^{13}C NMR the presence the following alkaloids voacangine hydroxyl indolenine, voacristine, voacristine hydroxyl indolenine, and 3-hydroxylcoronaridine. Presumably, all these alkaloids were present in the AF.

The anticancer activity of samples of *T. catharinensis* extract (Sample 1 and Sample 2) are presented in Figure 3. In this figure, growth bellow 100% up to zero is associated to the cytostatic activity of the extract, while negative growth represents the cytolytic activity of the extract. The dashed line is draw

to indicate when the extract can be considered active: (inhibition of growth is >50%). Figure 3 (a) shows that the extract sample collected over the extraction time interval of 60-120 min. exhibited only cytostatic effect. It is possible that cytolytic effects would be observed at higher concentration (> 250 μg/mL). Figure 3 (b) shows that the extract sample obtained over the depressurization (540-585min) step has anticancer activity that begins at 25 μg/mL and exhibits cytostatic and cytolytic effects: concentration dependent and not selective for the cell lines used. The comparison of the results in Table 1 with that of Figure 3 indicates that in spite of the larger content of voacangine of Sample 1 it did not inhibit the cell growth while Sample 2 did. thus, the voacangine content by itself cannot be associated to the anticancer activity. The content of total alkaloids in Sample 2 was larger (53.3%); therefore, it is probable that the anticancer activity is the result of the interaction of the entire group of alkaloids present in Sample 2.

These results confirm that the extract obtained by SFE maintained the anticancer activity, and suggest that the anticancer activity is associated to the group of alkaloids. Therefore, further studies are required to establish the compounds responsible for the anticancer activity.

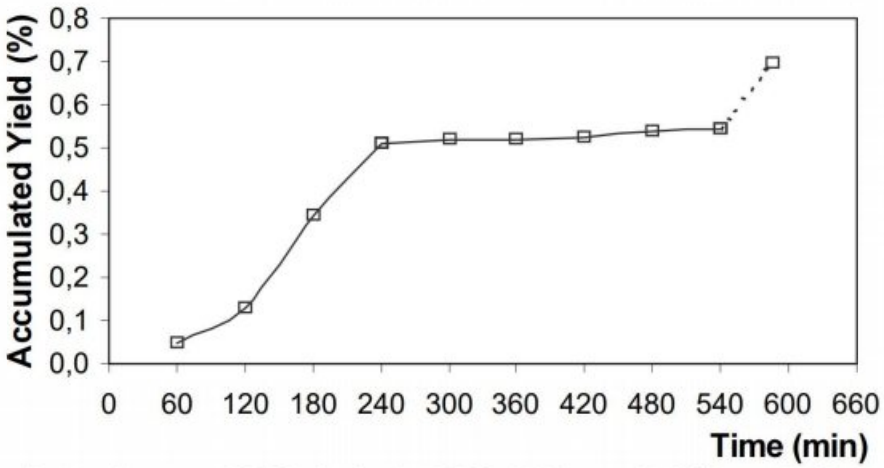

FIGURE 1. Overall extraction curve (OEC) obtained at 55 ºC, 300 bar and 4.6% (mass) of the cosolvent ethanol.

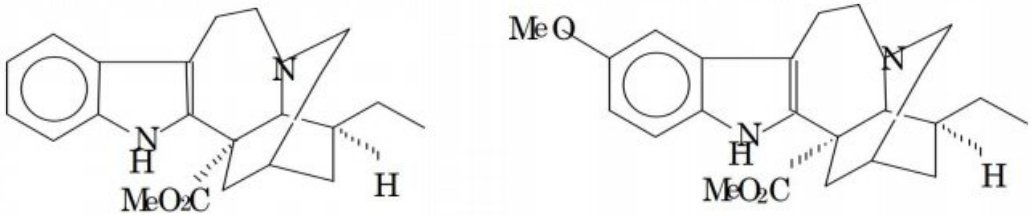

FIGURE 2. Indole alkaloids chemical structures from *T. catharinensis*

TABLE 1. Mass of crude extract and the percentages of alkaloidal fraction in the crude extract (AF/CE) and voacangine in the alkaloidal fraction (V/AF) for the samples collected over the extraction time intervals of 60-120 and 540-585 min.; SFE procedure done at 300 bar, 55 ºC and 4.6% (mass) of the cosolvent ethanol.

Sample	Extraction time interval, min	CE (mg/g)	AF/CE (%)	V/AF (%)
1	60 – 120	0.85	29.8	35.6
2	540 – 585	1.54	53.3	14.6

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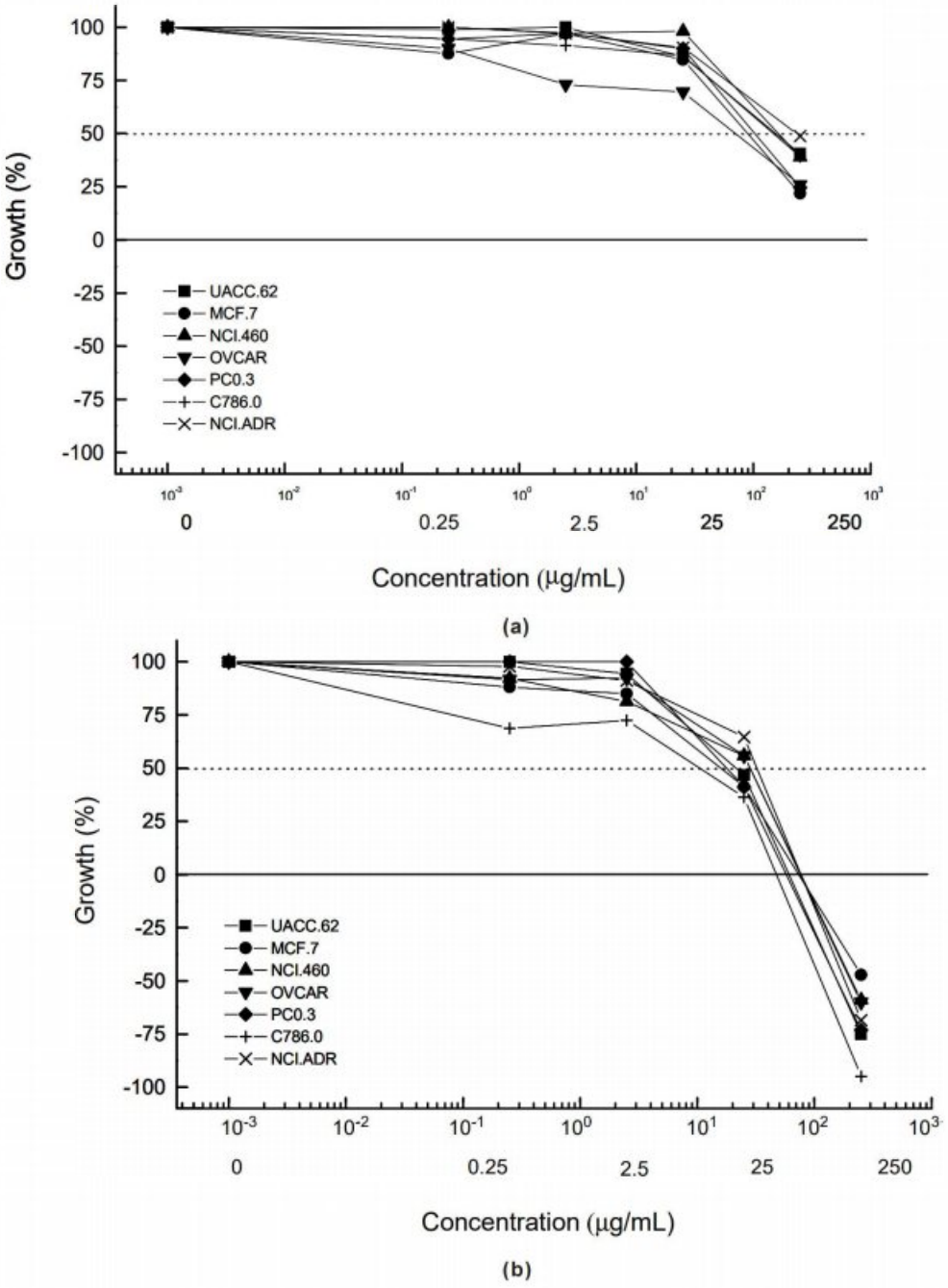

Figure 3. Anticancer activity as function of cancerous cellular ancestries for the samples of *T. catharinensis* extract collected over the time intervals (a) 60-120 min and (b) 540-585 min, at 300 bar, 55 ºC and 4.6% (mass) of the cosolvent ethanol. UACC.62 (melanoma), MCF.7 (breast), NCI.460 (lung), OVCAR (ovary), PC0.3 (prostate), C786.0 (kidney), and NCI.ADR (breast expressing the multidrug resistance phenotype).

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